GCCTTGAAGGGAGAAGGC-3'; nt 3562 through 3579), I-JS-S (5'-GAGAAGCTTGTAATGAATTGC-3'; nt −57 to −37 from the 5' base of exon B), F11CAS (5'-CAGACATCGGGTTCTTTGC-3'; nt 3716 through 3698), and F10BS (5'-TCCAGAAGGGTTTTCCTTG-3'; nt 3426 through 3444); the numbering of nucleotides is as in (*4*). *OAT*: OATE5-S (5'-GAGACTGCCTGTAAACTAGC-3'; nt 439 through 458), OATE9-AS (5'-ACCGTATGTGGACCCATGC-3'; nt 972 through 954), and OATE7-S (5'-GAGAGCTCTGCACCAGGCAC-3'; nt 748 through 767); the numbering is as in (*13*).
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29. PCR amplification of genomic DNA with primers F11CS and F11BAS was performed as described (*3*), except that denaturing, annealing, and extension times were extended to 1 min, 1.5 min, and 3 min, respectively.
30. We thank H. H. Kazazian, Jr., and A. F. Scott for critical review of the manuscript. Supported in part by NIH grants AR-41135, HG-00373, RR-00722, DK-44003, and HL-02815, the National Marfan Foundation, and an institutional research grant from the Johns Hopkins University School of Medicine. H.C.D. is a Ross Research Scholar. G.R.C. is a Merck Clinician Scientist.

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The Effect of Posttranslational Modifications on the Interaction of Ras2 with Adenylyl Cyclase

Yuichi Kuroda, Noboru Suzuki, Tohru Kataoka*

Ras proteins undergo a series of posttranslational modifications that are critical for their cellular function. These modifications are necessary to anchor Ras proteins to the membrane. Yeast Ras2 proteins were purified with various degrees of modification and examined for their ability to activate their effector, adenylyl cyclase. The farnesylated intermediate form of Ras2 had more than 100 times higher affinity for adenylyl cyclase than for the unprocessed form. The subsequent palmitoylation reaction had little effect. In contrast, palmitoylation was required for efficient membrane localization of the Ras2 protein. These results indicate the importance of farnesylation in the interaction of Ras2 with its effector.

The yeast *Saccharomyces cerevisiae* has two *RAS* genes, which encode proteins that are structurally, functionally, and biochemically similar to mammalian Ras oncoproteins (*1, 2*). The yeast Ras proteins are essential, positive controlling elements of adenylyl cyclase (*3, 4*). Yeast cells possessing the activated *RAS2* gene display abnormal phenotypes, including sensitivity to heat shock, sensitivity to nutritional starvation, and failure to sporulate (*3, 5*). Mammalian Ras proteins can substitute for yeast Ras proteins and activate yeast adenylyl cyclase (*2, 4*).

The Ras2 protein undergoes a series of posttranslational modifications (*6, 7*) that are common to members of the Ras protein family (*8*). The first stage of the processing consists of three modifications of a CAAX motif (C, cysteine; A, aliphatic; and X, any amino acid) at the COOH-terminus: (i) farnesylation of the cysteine residue (Cys^{319}); (ii) proteolytic cleavage of the amino acids AAX; and (iii) methyl-esterification of the new COOH-terminal cysteine. This first stage of processing converts the primary translation product into an intermediate form. The second posttranslational modification of Ras2 is acylation with palmitic acid on a cysteine residue (Cys^{318}) upstream of the CAAX motif. The first stage of processing is a prerequisite for the second stage.

Department of Physiology, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650, Japan.

*To whom correspondence should be addressed.

These modifications are essential for the anchoring of mammalian Ras proteins to the cytoplasmic membrane (*9, 10*) and for the transformation of NIH 3T3 cells (*9, 10*), induction of neuronal differentiation of PC12 cells (*11*), and induction of germinal vesicle breakdown in *Xenopus laevis* oocytes (*12*) by activated Ras. However, in vertebrate systems, the effect of the modifications on the activity of Ras proteins could not be examined separately from their effects on membrane anchoring. In budding yeast, an in vitro system exists for studying the interaction of Ras proteins with an effector. It has been shown that the unprocessed Ras2 protein from yeast produced in *Escherichia coli* can activate yeast adenylyl cyclase (*4, 13*). Thus, posttranslational modifications are not absolutely required for the interaction of Ras2 with its effector.

We purified various modified forms of Ras2 and observed their differential action on yeast adenylyl cyclase. The wild-type *RAS2* gene was cloned into a baculovirus transfer vector and overexpressed in Sf9 insect cells (*14*). Fully processed (mature) Ras2 was purified from a detergent extract of the insect cells (*15*). To purify the intermediate and the unprocessed forms of Ras2 proteins, we constructed *RAS2* genes with serine in place of Cys^{318} or Cys^{319} by site-specific mutagenesis and used these genes for overproduction of mutant Ras2 in insect cells (*16*). The three forms of Ras2 protein were purified almost to homogeneity and had apparent molecular sizes of 41 kD (unprocessed form) and 40 kD (intermediate and mature forms) (Fig. 1A). The three forms were characterized by incorporation of ^{3}H-labeled palmitic acid and by C4 reversed-phase chromatography (*6*) (Fig. 1). The incorporation of palmitic acid was observed only in the putative mature form. The elution positions of the three, purified forms of Ras2 in C4 reversed-phase chromatography and their apparent molecular sizes were identical to those reported for the respective forms produced in yeast cells (*6*). No difference was observed in the rate of guanosine triphosphate (GTP) binding and in the affinities for both GTP and guanosine diphosphate (GDP) among the three forms (*17*).

We next examined the ability of the three forms of Ras2 to activate yeast adenylyl cyclase. A crude membrane fraction of yeast cells harboring a plasmid that overexpresses the yeast adenylyl cyclase gene (*CYR1*) was assayed for adenylyl cyclase activity in the presence of Mg^{2+}, guanosine-5'-*O*-(3-thiotriphosphate) (GTP-γ-S), and various concentrations of each of the purified forms of Ras2 (*18*) (Fig. 2, A and C). Both the mature and the intermediate forms were 100 times more potent at activating adenylyl cyclase than the unprocessed form. The maximal extent of the activation caused by each of the three forms was similar. The activation by the unprocessed form was comparable to that obtained with Ras2 protein produced in *E. coli* (*4, 13, 19*). We assumed that the extent of activation reflected association with Ras2 protein and calculated the apparent dissociation constants (K_d's) from Scatchard plots to be about 3, 4, and 400 nM for the mature, intermediate, and the unprocessed forms, respectively.

To determine whether the observed differential action of the three forms resulted from enhanced association with adenylyl cyclase or with the cell membranes, we conducted a similar assay with adenylyl cyclase that was solubilized by extraction with 1.3 M NaCl (*20*) (Fig. 2, B and C). The soluble adenylyl cyclase was activated by the mature and the intermediate forms at concentrations similar to those that activated the membrane-bound adenylyl cyclase; the apparent K_d values were 3 nM for both of them. The unprocessed form was less effective. The apparent K_d was 1.6 μM, but the maximal extent of activation was comparable to that caused by the processed forms. Thus, the differential actions of the three forms do not appear to be explained by a difference in their ability to bind to membranes. The observed 100-fold increase in the apparent affinity for adenylyl cyclase resulted from the first stage of posttranslational processing, presumably the farnesylation of Ras2 protein. The subse-

quent palmitoylation of the protein appeared to have little effect.

We next examined the effect of the modifications on membrane localization of Ras2 in vitro. Each of the three forms of Ras2 was incubated with a crude membrane fraction from *ras2*$^-$ yeast cells overexpressing the *CYR1* gene. After incubation, Ras2 bound to the membrane was separated from the unbound protein by centrifugation. We measured the amounts of the bound and the unbound Ras2 protein by protein immunoblotting (*21*) (Fig. 3). A majority (>80%) of the mature form and about 30% of the intermediate form were recovered in the membrane, whereas most (>90%) of the unprocessed form remained in the supernatant. Thus, palmitoylation appeared to be required for efficient localization of Ras2 protein to the membrane. The observed association with the membrane presumably reflected an anchoring to lipid bilayers but not a specific binding to the adenylyl cyclase molecule, because an identical result was obtained with a membrane fraction from *ras2*$^-$ yeast cells having a defective *CYR1* gene (*17*). This was expected because the amount of adenylyl cyclase in the membrane was estimated to be much less than that of the added Ras2 protein even when *CYR1* was overexpressed (*17*).

To determine whether the observed effects of the mature and the intermediate forms of RAS2 really reflected their affinity for the adenylyl cyclase molecule, we tested the effects of these proteins on purified adenylyl cyclase. To purify Ras-responsive adenylyl cyclase, a segment containing the COOH-terminal 1421–amino acid residues of adenylyl cyclase was overexpressed in yeast cells as a fusion protein with glutathione-S-transferase (GST) (*22*). The NH_2-terminal 605 residues of adenylyl cyclase deleted in the fusion protein are dispensable for interaction with Ras proteins (*19*). Adenylyl cyclase solubilized from yeast cells overexpressing the fusion protein was activated by the three forms of Ras2 protein in a manner similar to that of the wild-type adenylyl cyclase (*17*). The apparent K_d values for Ras2 were 7 nM (mature and intermediate forms) and 200 nM (unprocessed form). The fusion protein was purified by affinity chromatography on glutathione-agarose and assayed for activation by the three forms of Ras2 protein (Fig. 4). The purified adenylyl cyclase formed a 450-kD complex containing an adenylyl cyclase–associated protein (CAP) that was 70 kD and at least two other proteins (*23*). Again, the mature and the intermediate forms had a higher apparent affinity (approximate K_d values of 8 and 14 nM, respectively) for the purified adenylyl cyclase complex than for the unprocessed form (K_d of 300 nM). Although we cannot exclude the possibility that a small amount of lipid is still attached to the purified adenylyl cyclase, these results, taken together with the result of the membrane-binding experiment, indicate that the effect of the first stage of posttranslational processing is to increase the affinity of Ras2 for the adenylyl cyclase complex.

In yeast cells, overexpression of the ex-

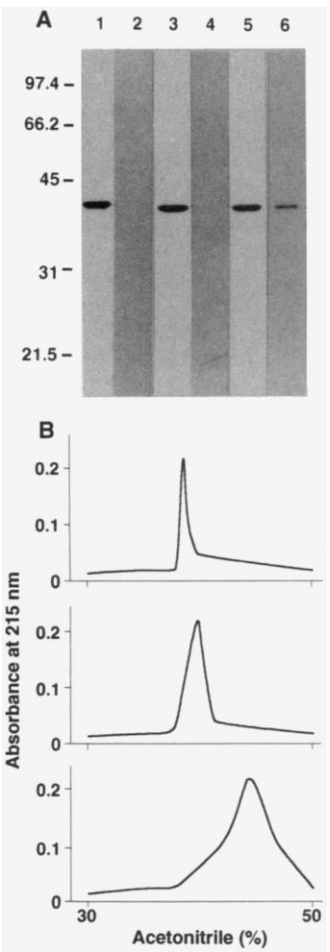

Fig. 1. Characterization of the three forms of Ras2 protein. (**A**) The three, purified forms of Ras2 produced in Sf9 cells (*15, 16*) were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (12% gel) (*29*) and detected by staining with Coomassie brilliant blue. Lane 1, unprocessed form; lane 3, intermediate form; and lane 5, mature form. In a separate experiment, the three forms were metabolically labeled in vivo with [9,10-^{3}H]palmitic acid (New England Nuclear) (*30*), purified, and analyzed by electrophoresis similarly. The incorporated radioisotopes were detected by fluorography (*31*). Lane 2, unprocessed form; lane 4, intermediate form; and lane 6, mature form. The molecular size markers are indicated at the left in kilodaltons. (**B**) Each of the three, purified forms of Ras2 protein was applied to a C4 reversed-phase column (AP-802 from YMC, Kyoto, Japan) and eluted with a linear gradient from 30 to 50% acetonitrile for 60 min at 1 ml/min (*6*). We monitored the elution patterns by measuring the absorbance at 215 nm. Top, unprocessed form; middle, intermediate form; bottom, mature form. Similar experiments in (A) and (B) performed on two separate occasions gave identical results.

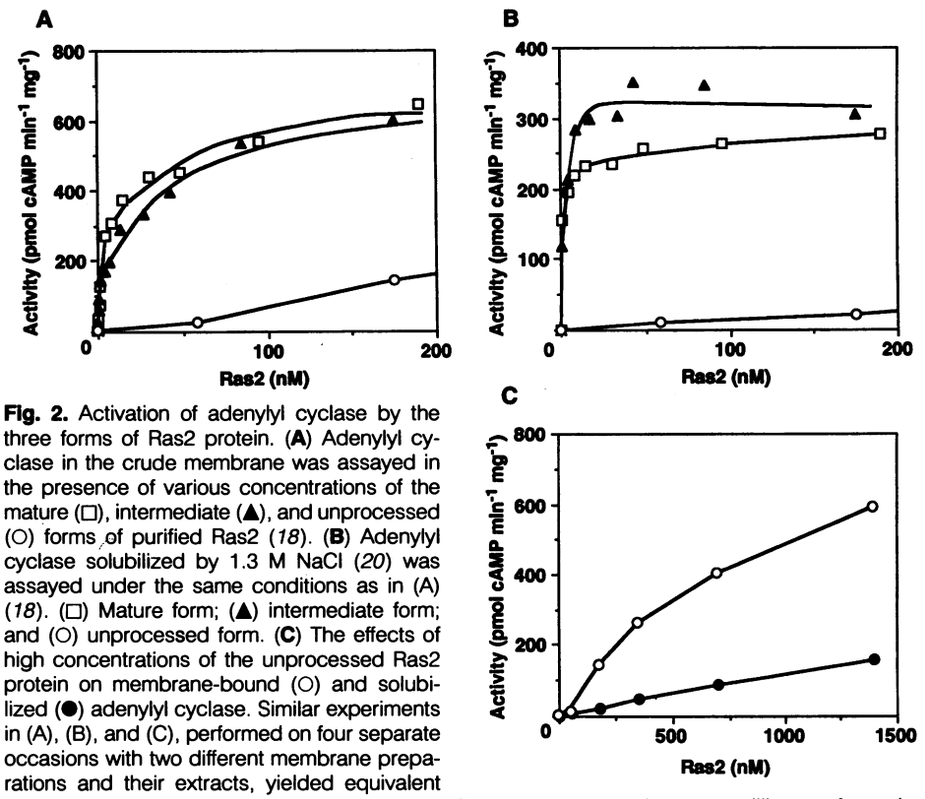

Fig. 2. Activation of adenylyl cyclase by the three forms of Ras2 protein. (**A**) Adenylyl cyclase in the crude membrane was assayed in the presence of various concentrations of the mature (□), intermediate (▲), and unprocessed (○) forms of purified Ras2 (*18*). (**B**) Adenylyl cyclase solubilized by 1.3 M NaCl (*20*) was assayed under the same conditions as in (A) (*18*). (□) Mature form; (▲) intermediate form; and (○) unprocessed form. (**C**) The effects of high concentrations of the unprocessed Ras2 protein on membrane-bound (○) and solubilized (●) adenylyl cyclase. Similar experiments in (A), (B), and (C), performed on four separate occasions with two different membrane preparations and their extracts, yielded equivalent results. Activity is measured in picomoles of cAMP production per minute per milligram of protein.

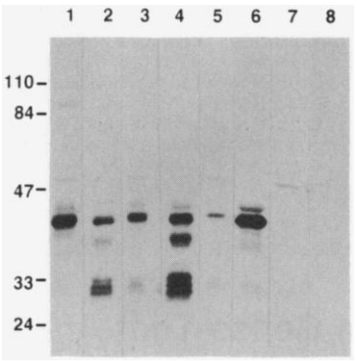

Fig. 3. Binding of the three forms of Ras2 to membranes. The three forms (10 pmol each) were incubated in the adenylyl cyclase assay buffer for 30 min at 30°C with the crude membrane fraction (30 μg) from the TK35-1 yeast cells harboring *YEP24-ADC1-CYR1* (*18*). Ras2 protein that bound to the membrane was separated from unbound protein by centrifugation at 21,000*g* for 90 min. One-tenth of the membrane-bound (lanes 1, 3, 5, and 7) and the unbound (lanes 2, 4, 6, and 8) material was separated by SDS-PAGE (12% gel) and subjected to protein immunoblotting with antibody to Ras2 (*21*). Lanes 1 and 2, mature form; lanes 3 and 4, intermediate form; lanes 5 and 6, unprocessed form; and lanes 7 and 8, no Ras2 protein. Results shown are representative of two experiments that gave equivalent results. Molecular size standards are indicated at the left in kilodaltons.

ogenous *RAS2* gene encoding the unmodified form is required to suppress the lethality brought about by a simultaneous loss of the endogenous *RAS1* and *RAS2* genes (*24*). An activated form of the protein, $Ras2^{Val19}$, without the CAAX motif did not localize on the membrane and did not confer sensitivity to nutritional starvation and galactose intolerance to yeast cells

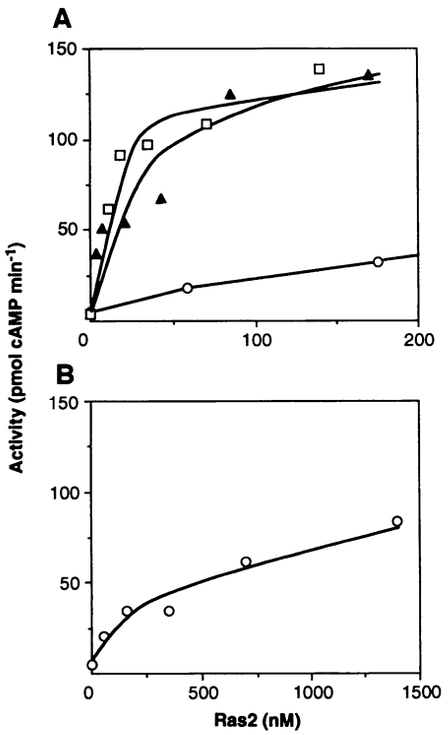

Fig. 4. Activation of purified adenylyl cyclase with the three forms of Ras2. (**A**) Adenylyl cyclase, expressed as a fusion protein with GST and purified by glutathione-agarose chromatography (*22*), was assayed in the presence of various concentrations of the mature (□), intermediate (▲), and unprocessed (○) forms of Ras2 as described (*18*). (**B**) Activation of adenylyl cyclase by high concentrations of the unprocessed form. Similar experiments in (A) and (B) performed on two occasions with different preparations of purified adenylyl cyclase yielded equivalent results.

(*24*). Mutations in subunits of farnesyl transferase, *ram1/dpr1* and *ram2*, abolished the membrane localization of Ras proteins and suppressed the $RAS2^{Val19}$ phenotype (*25*). These data have been interpreted to indicate that the loss of the action of unprocessed Ras2 protein resulted from the loss of its ability to bind to the plasma membrane where its effector is localized. Our results imply that the unprocessed Ras2 had its biological activity impaired by its reduced affinity for adenylyl cyclase.

The modifications of mammalian Ras are essential for the membrane localization and for the in vivo actions of Ras proteins (*9*, *11*, *12*). Site-specific mutagenesis of the COOH-terminal cysteine residues of H-Ras protein was used to examine the effect of the first stage of modification separately from that of palmitoylation (*10*). The percentage of the H-Ras protein that was localized to the membrane both in vivo and in vitro was approximately 90% for the mature, 8% for the intermediate, and 0% for the unprocessed forms. These results are similar to our data with Ras2. When the various modified forms of the activated H-Ras were examined for the ability to transform NIH 3T3 cells, the intermediate form retained some transformation activity, whereas the unprocessed form was totally inactive (*10*). These results may be compatible with our data. Artificial NH_2-terminal myristylation of the unprocessed H-Ras protein restored the ability to transform NIH 3T3 cells (*26*). This result was interpreted to mean that anchoring to the membrane was the critical function of the modifications on the action of Ras. However, these results may not be contradictory to our conclusion because a large amount of the myristylated Ras was expressed, which might have allowed the weakly active H-Ras protein to be functional.

The posttranslational modifications of K-Ras2B and other small GTP-binding proteins are essential for their interaction with a stimulatory GDP-GTP exchange factor (*27*). In the retinal phototransduction system, posttranslational modifications of the γ subunit of transducin are critical for its action and may increase the affinity of the βγ complex for the α subunit (*28*). The modifications of Ras2 may alter the conformation of Ras2 protein and facilitate its interaction with the effector molecule. Alternatively, an acceptor protein for the farnesyl group (or the carboxymethyl Cys^{319} or both) may exist in the adenylyl cyclase complex.

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15. *Spodoptera frugiperda* cells (Sf9) were infected with the recombinant nuclear polyhedrosis virus containing the *RAS2* gene and grown in Grace's Antheraea medium (Gibco, Grand Island, NY) containing yeastolate (0.033 g/ml) and lactalbumin hydrolysate (0.033 mg/ml) supplemented with fetal calf serum (10%) and 5 mM mevalonolactone for 3 days at 27°C. The cells were harvested and disrupted by sonication in buffer A [20 mM tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, and 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride] containing 10 μM leupeptin. After centrifugation at 10^5g for 1 hour, the sedimented material was washed with buffer A containing 50 mM NaCl and centrifuged again. The sediment was extracted twice with buffer A containing 50 mM NaCl and 60 mM 1-*O*-*n*-octyl-β-D-glucopyranoside (nOG). The resulting supernatant was applied to a MonoQ 5/5 column (Pharmacia, Uppsala, Sweden) and eluted with a linear gradient (20 ml) of 50 to 250 mM NaCl in buffer A containing 30 mM nOG as described [M. J. Page *et al.*, *J. Biol. Chem.* **264**, 19147 (1989)]. Ras2 protein in the eluate was detected by the [^{35}S]GTP-γ-S binding assay [A. Kikuchi *et al.*,

ibid. **263**, 2897 (1988)].
16. The 1.2-kb Hpa I fragment of the *RAS2* gene was cloned into a Hinc II cleavage site of M13tv18 phage DNA (Takara Shuzo, Kyoto, Japan) and subjected to oligonucleotide-directed mutagenesis with the gapped duplex method [W. Kramer and H.-J. Fritz, *Methods Enzymol.* **154**, 350 (1987)]. We used oligonucleotides GGGTGGCTCTTGTATT and TGGCTGTTCTATTATAAG to introduce substitutions of single amino acids of serine for Cys^{318} or Cys^{319}, respectively. The *RAS2* gene fragments carrying the mutations were transferred to *pBlueBac-βL1*, and we used the resulting plasmids to produce recombinant viruses (*14*). The intermediate form was purified as described (*15*). The unprocessed form was extracted without the detergent and purified in a similar manner.
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18. The structure of *YEP24-ADC1-CYR1*, which contains the complete wild-type *CYR1* gene under the yeast alcohol dehydrogenase I (*ADC1*) promoter, has been described (*19*). The plasmid was transformed into yeast strain TK35-1 (*MATα, leu2, his3, trp1, ura3, cyr1-2*, and *ras2::LEU2*) with Li acetate [H. Ito, Y. Fukuda, K. Murata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983)]. A crude membrane fraction was prepared from yeast cells (*13, 19*). The adenylyl cyclase assay was performed as described (*4, 13*), except that Ras2 was incubated with 0.1 mM GTP-γ-S for 30 min at 30°C in 20 mM tris-HCl (pH 7.4), 10 mM EDTA, 1 mM DTT, and 5 mM $MgCl_2$ before it was added to the reaction mixture. Crude membrane (11.6 μg) having 100 units [1 unit = 1 pmole of adenosine 3′,5′-monophosphate (cAMP) production per minute per milligram of protein] of Mn^{2+}-dependent adenylyl cyclase activity (*4, 13*) was assayed in a reaction mixture (100 μl) containing various concentrations of the preincubated Ras2 protein. We quantitated the three forms of Ras2 protein by measuring the saturated amounts of bound [^{35}S]GTP-γ-S.
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21. We prepared a polyclonal antiserum to Ras2 by immunizing rabbits with the Ras2 protein produced in *E. coli* as a fusion protein with the *Schistosoma japonicum* GST and purified by glutathione-agarose chromatography as described [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. All procedures with animals were performed in accordance with institutional guidelines. Ras2 protein was detected by protein immunoblotting as described [H. Towbin, T. Staehlin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)].
22. A segment of the *CYR1* gene corresponding to amino acid positions 606 to 2026 was fused to the COOH-terminus of GST and overexpressed in yeast cells under the *ADC1* promoter in yeast expression plasmid pAD1 (*13*). Adenylyl cyclase complex containing the GST-*CYR1* gene fusion product was solubilized by 1.3 M NaCl (*20*) and purified by glutathione-agarose chromatography (*21*). After elution with 5 mM glutathione in 50 mM tris-HCl (pH 8.0) and 0.15 M NaCl, adenylyl cyclase complex was separated from glutathione by chromatography (Sephadex G-25, Pharmacia) and used for the assay.
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Selection of Bacterial Virulence Genes That Are Specifically Induced in Host Tissues

Michael J. Mahan, James M. Slauch, John J. Mekalanos

A genetic system was devised that positively selects for bacterial genes that are specifically induced when bacteria infect their host. With the pathogen *Salmonella typhimurium*, the genes identified by this selection show a marked induction in bacteria recovered from mouse spleen. Mutations in all *ivi* (in vivo–induced) genes that were tested conferred a defect in virulence. This genetic system was designed to be of general use in a wide variety of bacterial-host systems and has several applications in both vaccine and antimicrobial drug development.

To understand the mechanisms by which a bacterial pathogen circumvents the immune system and causes disease, one has to identify those bacterial gene products that are specifically required for each stage of the infection process. The expression of most known virulence factors is regulated by environmental conditions in vitro that presumably reflect similar cues present in host tissues (*1*). The identification of many virulence factors has been dependent on, and limited by, the ability to mimic host environmental factors in the laboratory. We have developed a genetic system, termed IVET (in vivo expression technology), that does not rely on the reproduction of these environmental signals but rather depends on the induction of genes in the host. Thus, we selected bacterial genes that are highly expressed in host tissue but minimally expressed on normal laboratory media. Presumably, a subclass of genes that meet these selection criteria will encode products required for the infection process, including previously unidentified virulence factors.

The IVET strategy begins with a bacterial strain carrying a mutation in a biosynthetic gene that greatly attenuates growth in vivo; in this case, we used a *purA* auxotroph of *Salmonella typhimurium* (*2*). Growth in the host of such a mutant strain is then complemented by operon fusions to the same biosynthetic gene. The efficient selection for a $PurA^+$ phenotype in animal tissues (>10^8-fold selection) provides a means of positive selection for bacterial promoters that are expressed in host tissues by simply fusing them to a promoterless *purA* gene. In devising the system, we met three criteria: (i) construction of the fusions in single copy on the bacterial chromosome (to avoid complications that arise from using plasmids); (ii) no disruption of any chromosomal genes (to avoid the loss of any potential virulence factors); and (iii) provision of a convenient method to monitor the transcriptional activity of any given fusion both in vivo and in vitro.

We accomplished these goals by first constructing a synthetic operon consisting of a promoterless *purA* gene and a promoterless *lac* operon cloned into the broad host range suicide vector, pGP704 (*3*), resulting in pIVET1 (Fig. 1). The cloning of Sau 3AI *S. typhimurium* chromosomal fragments into the Bgl II site 5′ to the *purA* gene resulted in the construction of transcriptional fusions, where properly positioned *S. typhimurium* promoters drive the transcription of wild-type copies of *purA* and *lacZY*. Introduction of this pool of fusions into a Δ*purA* strain of *S. typhimurium* (*4, 5*) and selection for ampicillin resistance required the integration of the recombinant plasmids into the chromosome by homologous recombination with the cloned *S. typhimurium* DNA (Fig. 1). The product of this integration event generated a duplication of *S. typhimurium* DNA in which the native chromosomal promoter drove the *purA-lac* fusion, whereas the cloned promoter drove the expression of a wild-type copy of the gene.

The pattern of *lacZ* expression in a random pool of fusions was assessed in vitro on MacConkey Lactose indicator medium.

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.